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### (54) **Monoclonal antibody to a synaptophysin**

Monoklonaler Antikörper gegen ein Synaptophysin

Anticorps monoclonal dirigé contre une synatophysine

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- **D.C., USA pages 4137 - 4141 JAHN ET AL 'A 38,000-DALTON MEMBRANE PROTEIN (P38) PRESENT IN SYNAPTIC VESICLES'**
- **E1=Immunology by Roitt, Brostoff and Male, pub. Gower medical publications, section 8.1-8.5**

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**EP 0 528 663 B1**

**Description**

The present invention relates to a monoclonal antibody which specifically recognizes a synaptophysin.

A synaptophysin was found in a bovine brain by Wiedenmann et al. in 1985 (Cell, 41, 1017-1028), the synaptophysin being an acidic glycoprotein which is present on the membrane of a synaptic vesicle and has a molecular weight of about 38,000. On the other hand, a P-38 was found in a rat brain by Jahn et al. in 1985 (Proc. Natl. Acad. Sci. USA, 82, 4137-4141). However, when nucleotide sequences of the synaptophysin and the P-38 were analyzed, they were found to be identical proteins.

Since the synaptophysin and the P-38 locally exist in synaptic vesicles, they are used as detection markers for a neuroendocrine tumor which holds synaptic vesicles. The neuroendocrine tumor is formed in endocrine organs having synaptic vesicles such as a pituitary gland and a pancreas, but the tumor is metastasized to a organ other than the endocrine organ, which has no synaptic organs. If the synaptophysin is detected in an organ having no synaptic vesicles, it is understood that the organ holds the tumor and that the tumor is metastasized from the endocrine organ mentioned above. Thus, the synaptophysin specific monoclonal antibody of the present invention is useful to locate a primary focus of the neuroendocrine tumor.

At present, commercially available monoclonal antibody for the synaptophysin or the P-38 is monoclonal antibody which belongs to an immunoglobulin class G.

Since the conventional known monoclonal antibody for the synaptophysin belongs to an immunoglobulin class G, only two binding sites for antigen and complement are present within each molecule. For this reason, in order to quantitatively analyse a very small amount of synaptophysin in tissues in accordance with a complement fixation test, a large amount of the antibody is required, resulting in inconvenience.

Production of an anti-synaptophysin monoclonal antibody SY38 and an anti-synaptophysin polyclonal antibody, the subclass of the anti-synaptophysin monoclonal antibody SY38 being IgG is known by EP-A-0431567.

Hybridomas selected to produce IgM and IgG class monoclonal antibodies is disclosed by WO-A-8304313.

The present invention has been made in consideration of the above situation, and the object of the invention is to make it possible to perform the quantitative analysis of the synaptophysin in accordance with a complement fixation test, by using a small amount of the antibody, even if the amount of the synaptophysin in a biological tissue is very small. More specifically, the object of the present invention is to provide a synaptophysin specific monoclonal antibody belonging to an immunoglobulin class of M.

According to the present invention, there is provided the hybridoma RB2-4 FERM BP-3944, and the monoclonal antibody produced by the hybridoma RB2-4 FERM BP-3944. A pharmaceutical composition containing the monoclonal antibody is also provided.

The monoclonal antibody of this invention was obtained by using an extract of a rat brain as an immunogen, the monoclonal antibody belonging to an immunoglobulin class M, and the monoclonal antibody having specific reactivity for a synaptophysin or P-38.

The monoclonal antibody according to the present invention has specific reactivity for a protein expressed from a gene comprising the nucleotide sequence represented by sequence No. 1 in the sequence listing. (wherein A, C, G, and T represent adenine, cytosine, guanine, and thymine, respectively).

This invention can be more fully understood from the following detailed description when taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a photograph showing the SDS-PAGE development pattern of a rat brain homogenate and specificity of a monoclonal antibody of the present invention; and

Figs. 2A to 2B are views showing a homology between the nucleotide sequence of a rat synaptophysin gene obtained and ascertained by the present invention and the nucleotide sequence of a known rat synaptophysin gene.

The present invention will be described in detail below.

Preparation of a monoclonal antibody according to the present invention is explained first.

The monoclonal antibody can be prepared in accordance with a conventional monoclonal antibody technique. That is, an antibody producing cell collected from a mammalian animal immunized with a rat brain extract is fused with an appropriate tumor cell (e.g. a myeloma) of an animal, to prepare a hybridoma producing an objective antibody. This hybridoma is cloned and cultured to obtain a monoclonal antibody according to the present invention.

More specifically, a monoclonal antibody according to the present invention can be prepared in accordance with the following procedures.

#### a) Preparation of Antibody Producing Cell

In order to obtain a hybridoma producing a monoclonal antibody according to the present invention, a mammalian

animal is immunized using a rat brain extract.

As the mammalian animal for obtaining a cell producing a monoclonal antibody according to the present invention, a general laboratory animal such as a mouse, a rat, a rabbit, and a guinea pig can be used, but the mouse is preferably used.

As a method of immunizing a mammalian animal, a method of injecting a rat brain extract as an immunogen into, e.g., a mouse intraperitoneally or subcutaneously, can be used. A dosage per injection is preferably 0.2 ml/mouse of the immunogen, which is used for injection as a mixture with an equivalent amount of an adjuvant. This injection is repeated every one or two weeks and performed several times. Final immunization is performed by injecting 0.2 to 0.4 ml/mouse of the immunogen intravenously or intraperitoneally without mixing it with the adjuvant. A spleen of the mouse is excised 3 to 4 days after the final immunization injection, thereby obtaining an antibody producing cell.

#### b) Preparation of Tumor Cell

A tumor cell to be fused with the antibody producing cell is prepared as follows.

As a tumor cell according to the present invention, a myeloma cell is generally used. The origin of the tumor cell is not limited to a specific one. Any cell line derived from a mammalian animal such as a mouse, a rat, a rabbit, or human can be used. Among them all, a mouse cell line is preferably used. A tumor cell line having a suitable selection marker such as hypoxanthine guanine phosphoribosyl transferase deficiency (HGPRT<sup>-</sup>) or thymidine kinase deficiency (TK<sup>-</sup>) is generally used. Examples of this cell line are P3-X63-Ag8, P3-X63-Ag8-U1, and SP20-Ag8-6.5.3. All these tumor cells are 8-azaguanine-resistant cell lines and cannot be grown in an HAT medium (containing hypoxanthine, aminopterin, and thymidine).

#### c) Cell Fusion

The antibody producing cell is fused with the tumor cell.

As a medium used in cell fusion, a medium obtained by adding 10% CS (calf serum), 5% FCS (fetal calf serum) + 5% CS, or 10% FCS to a generally used essential medium such as an Eagle's minimum essential medium (MEM), a Dulbecco's modified Eagle MEM, or Rosewell Park Memorial Institute (RPMI) 1640 can be used. Any combination of the serum and the essential medium mentioned above can be used for subculturing parent cells. However, when a hybridoma is to be prepared, a medium containing 10% FCS is preferable.

The cell fusion is performed by mixing the two parent cells, that is the tumor cells (e.g., myeloma) and the immunized splenocytes (i.e., antibody producing cells) at a ratio of 1 : 5 to 1 : 20 in the presence of a fusion accelerator. Examples of the fusion accelerator is an HVJ (Hennagglutinating Virus of Japan) and polyethylene glycol (PEG). 30% - 50% PEG 1500 is most preferable as the fusion accelerator.

#### d) HAT Selection of Hybridoma

A hybridoma is selected in the HAT medium. The fused cells obtained by procedure c) are appropriately diluted with e.g., RPMI1640 containing 20% FCS and are seeded in a microculture plate (normally a 96- or 24-well plate) at about  $10^4$  to  $10^6/100 \mu\text{l}$ /well. The HAT selection medium is then added to each well, and the culture is performed while the medium is changed with a new one generally every one to two days.

When an 8-azaguanine resistant cell line is used as tumor cells, nonfused myeloma cells and myeloma-myeloma fused cells die in the HAT medium within about seven days. Also splenocytes cannot be grown over two weeks in-vitro, since they are normal cells and have a predetermined life time. Therefore, all cells growing 7 to 10 days after starting the culture are regarded as spleen-myeloma fused cells.

#### e) Screening of Hybridoma

The hybridomas selected by procedure d) are screened. The clones of hybridomas which secrete an objective antibody are picked up by a known method such as an ELISA (Enzyme-linked immunosorbent assay), or a immunoblotting method using a culture supernatant of each well in which hybridomas are grown.

#### f) Cloning

An objective hybridoma obtained by the screening is cloned. Two or more types of hybridomas which produce different antibodies may be grown in each well. For this reason, cloning may be performed using a limiting dilution analysis to obtain single hybridoma clones which produce the objective monoclonal antibody.

## g) Acquisition of Monoclonal Antibody

The hybridoma obtained in procedure f) is cultured to obtain the objective monoclonal antibody.

This hybridoma can be cultured in vitro or in vivo. When it is cultured in-vitro, a medium used in this culture can be the above-mentioned medium obtained by adding CS or FCS to the normal medium. After the hybridoma is cultured in this medium for three to five days, the objective antibody can be acquired from the supernatant of the medium. When it is cultured in-vivo, a mineral oil such as pristane(2,6,10,14-tetramethylpentadecane) is intraperitoneally injected into an animal whose species is same as the origin of the myeloma. After a lapse of at least one week, the hybridoma is intraperitoneally injected. After 7 to 14 days, the abdominal dropsy stayed is sampled to obtain the objective antibody.

Now a gene which expresses synaptophysin recognized by the monoclonal antibody according to the present invention will be described below.

The gene can be obtained by the following procedures. That is, cDNA library prepared from a rat brain using a  $\lambda$ gt11 vector is transfected into appropriate host cells, thereby expressing the synaptophysin gene. A protein produced by the host cells is screened using an antibody which specifically binds to the rat synaptophysin, thereby selecting a vector in which an objective cDNA fragment is inserted. The selected vector is amplified to obtain the synaptophysin gene.

More specifically, the gene which expresses the synaptophysin recognized by the monoclonal antibody according to the present invention can be prepared by the following procedures.

## h) Screening

Host cells (e.g., E.coli) are cultured in an appropriate medium, and a rat brain cDNA library which has been inserted in the  $\lambda$ gt11 vector available from Clontech Inc. is transfected in the host cells. The cDNA library may be transfected in such a way that, for example, the E.coli is cultured in an appropriate medium such as LBM medium, and then the E.coli suspension is infected with the cDNA-inserted phage vector suspension in the ratio of  $5 \times 10^4$  pfu of the phage vector suspension to  $1 \mu\ell$  of the E.coli suspension. The resultant mixture is spread on an agar plate and cultured thereon to form plaques. An IPIG soaked N.C. (membrane filter) is placed on the formed plaques, and then incubated. A produced protein by the expression of the cDNA inserted in the vector is transferred to the nitrocellulose membrane.

The anti-synaptophysin monoclonal antibody is reacted with the protein on the nitrocellulose membrane, and then a labeled antibody capable of specifically binding to this anti-synaptophysin antibody is reacted. After the reaction, a treatment required for detecting the marker of the antibody is performed, thereby detecting a spot in which the marker is present. By searching the plaque corresponding to this spot, a vector containing an objective gene fragment for rat synaptophysin can be obtained.

## i) Amplification of Vector Containing Rat Synaptophysin Gene

The synaptophysin gene screened by procedure h) is amplified.

The vector confirmed to contain a rat synaptophysin gene fragment can be amplified by culturing the host cell transfected with the vector in accordance with a plate lysate (stock) method or a liquid culture method.

## j) Preparation of Rat Synaptophysin Gene

A target gene is prepared from the amplified vector.

Only a phage containing a rat synaptophysin gene fragment is recovered from the host cells. The recovered phage is lysed using chloroform and then proteins are removed with e.g., phenol. After proteins are removed, a DNA containing a rat synaptophysin gene fragment is obtained by precipitating with ethanol.

As described previously, the monoclonal antibody of the present invention can be specifically bounded to synaptophysin. Thus, a pharmaceutical composition containing the monoclonal antibody of the present invention and a pharmaceutically acceptable carrier or diluent is useful for the diagnosis of a neuroendocrine tumor which utilizes the presence of synaptophysin as a marker.

For making the diagnosis possible, it is necessary for the monoclonal antibody of the present invention to be labeled. Any marker can be used as far as the marker can be detected after incorporation into a body. However, a radioisotope can be desirably used as a marker because an imaging diagnosis utilizing a scintigraphy method, which is described later, is most popular nowadays. Any radioisotope can be used as a marker depending on the half-life period, kind of radiation, object of diagnosis, etc., though it is desirable to use a radioisotope which is likely to be stored in the desired internal organ. Specific examples of the radioisotopes used as markers include, for example,  $^{99m}\text{Tc}$ ,  $^{111}\text{In}$ ,  $^{123}\text{I}$ ,  $^{131}\text{I}$ ,  $^{201}\text{Tl}$ ,  $^{75}\text{Se}$ ,  $^{169}\text{Yb}$  and  $^{67}\text{Ga}$ .

Any kind of carrier or diluent, which is pharmaceutically acceptable and, thus, used in general, can be used for

preparation of the pharmaceutical composition of the present invention. It is also possible to use a plurality of carriers or diluents in combination. It is desirable to use a sterile and aqueous isotonic suspension or solution including, for example, a physiological saline and a phosphate buffer physiological saline.

The pharmaceutical composition of the present invention can be administered by non-oral administration such as subcutaneous administration, intramuscular administration, intravenous administration, or intraperitoneal administration.

The pharmaceutical composition of the present invention can be administered in an amount of 1 to 100 mg/kg, desirably 1 to 10 mg/kg, of a mammal weight, though the amount of administration depends on the age and weight of a patient, method of administration, etc.

The pharmaceutical composition of the present invention can be prepared by a standard method.

Let us describe briefly the diagnostic method using the pharmaceutical composition of the present invention.

A scintigraphy method is most generally employed nowadays for the diagnosis. In the scintigraphy method, an isotope generating a  $\gamma$ -ray is administered into a body. A predetermined period of time later, the distribution of radioactivity of a desired organ or entire body is measured by a scinti scanner or a scinti camera, and the radioactivity distribution thus measured is displayed on a screen as a picture image by fully utilizing an electronic computer. Of course, the displayed picture image is used for the diagnosis for detecting a diseased portion of an organ, i.e., high or low isotope concentration region.

Specifically, a predetermined time after administration of an effective amount of the pharmaceutical composition, a picture image of an organ having a diseased portion (an organ which originally does not have synaptic vesicles), is depicted on a screen, making it possible to perform diagnosis to determine whether the diseased portion is a neuroendocrine tumor or not.

When the monoclonal antibody according to the present invention is used, only synaptophysin among proteins which are transferred to e.g., nitrocellulose, adsorbed in an ELISA plate, or contained in a tissue preparation such as a tissue slice can be easily detected. As its assay method, an indirect enzyme immunoassay or indirect fluorescent antibody technique used in combination with an anti-mouse IgM antibody labeled with an enzyme or a fluorescent dye is exemplified.

The monoclonal antibody according to the present invention belongs to an immunoglobulin class M and has 10 binding sites for antigen or complement within one molecule. When a complement fixation test using the monoclonal antibody according to the present invention is performed, a higher sensitivity can be obtained and a very small amount of rat synaptophysin can be quantitatively analyzed by a smaller amount of the antibody as compared with the analysis using a monoclonal antibody of the conventional IgG class.

It is also possible to perform diagnosis to determine whether the neuroendocrine tumor is metastatic or not by using a pharmaceutical composition containing the monoclonal antibody of the present invention. Further, in the case of using the pharmaceutical composition of the present invention, a diseased portion can be detected more easily by utilizing a scinti scanning method and an electronic computer than in the case of conventional diagnosis using X-rays.

Examples of the present invention will be described in detail below.

#### Example 1

#### Preparation of Monoclonal Antibody for Proteins of Rat Brain

##### (1) Preparation of Immunogen

The following preparation operations were performed at 0 to 4°C, unless otherwise specified. A 0.5 mM protease inhibitor PMSF was added to water and solutions, as needed.

3-fold volume of a buffer solution A (0.32 mM sucrose, 20 mM tris-citric acid buffer solution) was added to a rat brain, and the rat brain was homogenized. The homogenate itself may be used as an immunogen. In Example 1, however, after the homogenate was filtered through a nylon mesh (mesh sizes: 133  $\mu$ m and 77  $\mu$ m), the filtered homogenate was layered on an equal volume of buffer solution A and was centrifuged at 1,000 g for 10 minutes. After the centrifugation, the supernatant was recovered. This supernatant was centrifuged at 10,000 g for 30 minutes, and the resultant precipitate was collected (the extract obtained as the precipitate is called a P2 fraction hereinafter). The supernatant obtained by the second centrifugation was further centrifuged at 100,000 g for 150 minutes to collect a precipitate (the extract obtained as this precipitate is called a P3 fraction hereinafter). These fractions were used as an immunogen singly or mixed at an appropriate ratio to obtain an immunogen.

##### (2) Immunization of Animal

The immunogen prepared in operation (1) and a complete Freund adjuvant in equal volumes were charged in 1-ml

syringes. The syringes were connected with needles to stir the immunogen and the complete Freund adjuvant until the immunogen was emulsified with the adjuvant. This emulsion was intraperitoneally injected into a mouse to perform immunization. That is, an experiment was conducted as follows. Basically, the second immunization was performed two weeks after the first immunization. Booster was performed two weeks after the second immunization, thereby completing immunization. Cell fusion was performed three days after the booster. If the number of days had to be adjusted, booster was delayed to adjust the number of days. The antibody titer was assayed such that blood was sampled from the tail vein of the mouse two to seven days after the immunization and immunoblotting (to be described later in detail) was performed using its serum.

### (3) Cell Fusion

The mouse immunized by operation (2) was sacrificed by dislocating the cervical vertebrae thereof to take out its spleen. The spleen was placed in a 6-cm dish in which 5 ml of RPMI was placed, and an extra fat was removed from it. The spleen was washed in two 6-cm dishes each containing 5 ml of the RPMI. The spleen was rubbed onto a folded 5 cm square stainless net with pincettes to separate cells. The individually separated cells were transferred to a centrifuge tube and were washed with 10 ml of the RPMI twice by the centrifugation. 0.17 M  $\text{NH}_4\text{Cl}$  was added to the precipitated cells to hemolyze the cells in ice for 5 minutes. 5 ml of RPMI were added to the cells after the hemolysis and the cells were centrifuged at 1,600 rpm for 5 minutes. The precipitated cells were washed with RPMI and were messed up to have a volume of 20 ml, thereby obtaining a splenocyte suspension.

On the other hand, myeloma in a logarithmic growth phase in an amount corresponding to 2 to 4 dishes were collected by a centrifugation at 1,200 rpm for 5 minutes. The collected myeloma were washed twice with serum-free RPMI and were finally suspended in 10 ml of the RPMI to prepare a myeloma suspension. The numbers of cells contained in the splenocyte suspension and the myeloma suspension were counted, and the myeloma suspension was added to the splenocyte suspension such that the ratio of the number of myeloma to the number of splenocytes was set to be 1/5 to 1/20. The resultant mixture was centrifuged for 5 minutes, the supernatant was removed, and the splenocytes and the myeloma were suspended. All of 0.3 ml of 50% polyethylene glycol 1500 (in 75 mM Hepes) were quickly added to the cells and the cell suspension were immediately stirred well. While stirring was continued, the RPMI was added to the suspension. The resultant suspension was centrifuged at 1,000 rpm for 5 minutes. 50 ml of an HAT medium (an RPMI-FCS containing hypoxanthine having a final concentration of  $1 \times 10^{-7}$  M, aminopterin having a final concentration of  $4 \times 10^{-4}$  M, and thymidine having a final concentration of  $16 \times 10^{-4}$  M) were added to the precipitate. The resultant suspension was seeded in two 24-well plates in an amount of 500  $\mu\text{l}$ /well and in about three 96-well plates in an amount of 100  $\mu\text{l}$ /well. After four to five days, the HAT medium was added to the 24-well plates in an amount of about 1 ml/well and to the 96-well plates in an amount of about 100  $\mu\text{l}$ /well. As a result, it was found that hybridomas grew in almost all wells within about a week. The hybridomas were further cultured, and their supernatants were used for screening according to the immunoblotting method.

### (4) Selection and Cloning of Hybridoma

The immunogen prepared in operation (1) was diluted such that a total amount of proteins became several tens of mg/ml, and the resultant diluted solution served as an antigen solution. After the SDS-PAGE of the antigen was performed, the proteins were transferred to a nitrocellulose membrane by electrophoresis in accordance with a method proposed by Towbin et al. (1979). The nitrocellulose membrane was blocked using an appropriate blocking agent such as Block Ace (available from Dainippon-Pharmaceutical Co.) for an hour to overnight. The blocked nitrocellulose membrane was incubated together with the culture supernatant as a primary antibody for screening. Thereafter, the nitrocellulose membrane was washed with TBS three times each for 15 minutes. The nitrocellulose membrane was incubated and reacted with a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody. The reacted nitrocellulose membrane was washed with TBS three times each for 15 minutes. A color-developing solution obtained by adding 1/100-fold volume of 1%  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 1/100-fold volume of 1%  $(\text{NH}_4)_2\text{Ni}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ , and 1/500-fold volume of a hydrogen peroxide solution to 100  $\mu\text{g}/\text{ml}$  of diaminobenzidine tetrahydrochloride in 50 mM tris-HCl (pH 7.5) was used to detect a product of color-development reaction. After satisfactory coloring was performed, the nitrocellulose membrane was washed with distilled water and was dried. As a result of a search operation mentioned above, wells from which culture supernatants having a high activity were obtained, were selected. The hybridomas of these wells were cloned by the limiting dilution analysis using mouse thymocytes as feeder cells, thereby obtaining one clone. The hybridoma RB2-4 thus obtained is deposited with the Fermentation Research Institute, the Agency of Industrial Science and Technology, located at 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan and assigned the deposit number FERM BP-3944. The hybridoma was cultured to obtain a monoclonal antibody. This monoclonal antibody was named RB2-4.

An examination was performed using a Sub Isotyping Kit available from Amersham, Inc. The subclass of this

antibody was found to be an IgM.

## Example 2

### Examination of Reaction Specificity of RB2-4

The reaction specificity of RB2-4 was examined by immunoblotting as in Example 1, and the examination result is shown in Fig. 1.

Fig. 1 is a photograph showing the development pattern of the previously described brain homogenate by the SDS-PAGE and the binding position of RB2-4 by immunoblotting. Referring to Fig. 1, reference numeral 1 denotes a stain pattern of a molecular weight marker by a CBB; 2, a stain pattern of a rat brain homogenate by the CBB; and 3, a result of immunoblotting of the rat brain homogenate by using RB2-4. As can be apparent from Fig. 1, RB2-4 is reacted with a band of a single protein having a molecular weight of about 40 kD.

## Example 3

### Isolation of Rat Synaptophysin Gene

#### (1) Screening of Plaque by using Monoclonal Antibody RB2-4

*E. coli* (Y1090 ( $\Delta$ lacU169proA+ $\Delta$ lon araD139strAsupF[trpC22::Tn10] (pMC9\*), mcrA-, mcrB+)) was cultured overnight using an LBM medium (containing 0.2% maltose and 50 mg/ml Ampicillin). A cDNA library (available from Clontech Inc.) constructed from a rat brain using a  $\lambda$ gt11 vector was diluted to about  $4 \times 10^5$  pfu. This diluted suspension was mixed with 0.6 ml of an *E. coli* suspension obtained by the above culture, and the mixture was incubated at room temperature for 20 minutes. On the other hand, a top agarose (e.g., LBM agarose) was treated in an autoclave and incubated in a molten state at 55°C. The treated top agarose was mixed with the above mixture, and the resultant mixture was stirred quickly. The resultant mixture was spread on a bottom agar (e.g., 0.8% LMB agar) spread on a plate in advance. After the top agarose was solidified, the solid body was incubated at 42°C for 3.5 hours to form a plaque. A nitrocellulose membrane was placed on the plaque and was incubated at 37°C for 3.5 hours, thereby expressing the cDNA transfected in the *E. coli*. This nitrocellulose membrane had been immersed in 10 mM IPTG (isopropylthiogalactopyranoside) and was dried in advance. Thereafter, the nitrocellulose membrane was washed with TBS and was used for subsequent screening by an antigen-antibody reaction.

Screening using RB2-4 was performed as follows. The nitrocellulose membrane was blocked using TBS containing 3% gelatin at room temperature for an hour to overnight. After the blocked nitrocellulose membrane was reacted with the monoclonal antibody (primary antibody) used in screening, the nitrocellulose membrane was washed with TBS three times each for 15 minutes. Subsequently, the nitrocellulose membrane was reacted with a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (secondary antibody). The nitrocellulose membrane was then washed with TBS three times each for 15 minutes. A color-developing solution obtained by adding 1/100-fold volume of 1%  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 1/100-fold volume of 1%  $(\text{NH}_4)_2\text{Ni}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ , and 1/500-fold volume of hydrogen peroxide solution to 100  $\mu\text{g}/\text{ml}$  diaminobenzidine tetrahydrochloride in 50 mM tris-HCl (pH 7.5) was used to detect a product of color-development reaction. After satisfactory coloring was performed, a plaque corresponding to the stained spot was searched to obtain a phage having an objective gene fragment.

#### (2) Proliferation of Phage Having Rat Synaptophysin Gene and its Complementary Strand

The phage having the rat synaptophysin gene and its complementary strand obtained in operation (1) was proliferated in accordance with the following procedures of the plate lysate method. *E. coli* (Y1088 ( $\Delta$ lacU169supEsupFhdsR-hsdM+metBtrpRtonA21[proc::Tn5] (pMC9), mcrA-mcrB+)) was cultured overnight using LBM medium (containing 0.2% maltose and 50 mg/ml Ampicillin). 0.1 ml of SM buffer solution containing  $10^5$  to  $10^6$  pfu of the phage was added to the *E. coli* suspension and was incubated at room temperature for 10 minutes. On the other hand, a top agarose (e.g., LBM agarose) was treated in an autoclave and incubated in a molten state at 55°C. The treated top agarose was mixed with the above mixture, and the resultant mixture was stirred quickly. The resultant mixture was spread on a bottom agar (e.g., LMB agar) spread on a plate in advance. After the top agarose was solidified, the solid body was put into a vinyl bag and cultured at 37°C for 6 to 7 hours. After the *E. coli* was lysed, 5 ml of SM buffer solution and some drops of chloroform were added to the solid body. The solid body was incubated overnight at 4°C while it was held horizontally so that SM buffer solution perfectly covered the surface thereof. As a result, a phage suspension in which a phage having the rat synaptophysin gene and its complementary strand was suspended was obtained.

## (3) Preparation of Rat Synaptophysin Gene and its Complementary Strand

5  $\mu\text{g}/\text{mL}$  of RNaseA and 5  $\mu\text{g}/\text{mL}$  of DNaseI were added to the phage suspension obtained in operation (2), which comprises the rat synaptophysin gene and its complementary strand and the resultant suspension was incubated at 37°C for 30 minutes. An equal volume of polyethylene glycol PEG 6000-2M NaCl solution was mixed with the above suspension and incubated at 0°C for an hour. The resultant mixture was centrifuged at 4°C, 3,000 rpm for 20 minutes, and the supernatant was perfectly removed. The resultant precipitate was suspended in 0.5 mL of SM buffer solution, and the suspension was transferred to an eppendorf tube and was centrifuged at 8,000 rpm for 2 minutes. The obtained supernatant was transferred to another eppendorf tube. EDTA and SDS were added to this supernatant so that final concentration of EDTA and SDS is 10 mM and 1% respectively, and the mixture was incubated at 65°C for 15 minutes. Thereafter, phenol extraction, phenol-chloroform extraction, and chloroform extraction were performed once, twice, and once, respectively. In the phenol extraction or phenol-chloroform extraction, phenol saturated with TE (tris-EDTA buffer solution) was used. 50  $\mu\text{L}$  of 3M sodium acetate and 1 mL of ethanol were added to the aqueous layer obtained by these extraction operations, and the mixture was incubated at -80°C for 15 minutes and was centrifuged at 15,000 rpm for 5 minutes. After the centrifugation, the supernatant was removed, and the resultant residue was washed with cooled 70% ethanol and was dried. The dried product was dissolved in 50  $\mu\text{L}$  of TE to obtain a phage DNA having the rat synaptophysin gene and its complementary strand.

The rat synaptophysin gene and its complementary strand could be taken from the phage DNA by using restriction enzymes (e.g., EcoRI).

In addition, only the rat synaptophysin gene and its complementary strand were obtained from the phage DNA digested by e.g., EcoRI in accordance with the following procedures. The phage DNA digested by the EcoRI was subjected to electrophoresis using a 1% agarose gel having a low melting point in a buffer system containing ethidiumbromide and TAE (EDTA-tris acetic acid buffer solution). After this electrophoresis, a target band of 0.8 kbp was cut out under UV radiation by a transilluminator and the target band were transferred to an eppendorf tube. A gel containing the target band were melted at 65°C and were added with distilled water. The resultant mixture was gradually cooled. After phenol extraction and phenol-chloroform extraction were performed twice and once, respectively, DNA was recovered by ethanol precipitation. In these extractions, phenol saturated with TE was used.

When the nucleotide sequence of the DNA was determined by a dideoxy method, it was found that the DNA comprised a nucleotide sequence represented by sequence No. 1 in a sequence listing. Sequence No. 1 represents the nucleotide sequence of the rat synaptophysin gene obtained in the present invention, and its sequence was represented in a direction toward the 5'-terminus. Herein, A, C, G, and T represent adenine, cytosine, guanine, and thymine, respectively.

Sequence No. 2 in the sequence listing represents the nucleotide sequence of the known rat synaptophysin gene. In this sequence listing, the nucleotide sequence was represented from No. 721 to No. 1,501 from the 5'-terminal to the 3'-terminal. The nucleotide sequence represented by sequence No. 1 according to the present invention corresponds to base Nos. 726 to 1,486 of the known synaptophysin gene.

The homology between the nucleotide sequence of the rat synaptophysin gene obtained in the present invention and the nucleotide sequence of the known rat synaptophysin gene is shown in Figs. 2A to 2B. The upper row represents the nucleotide sequence of the rat synaptophysin gene obtained by the present invention, and the lower row represents the nucleotide sequence of the known rat synaptophysin gene. An asterisk \* indicates an identical base between these base sequences. A, C, G, and T represent adenine, cytosine, guanine, and thymine, respectively; and a mark represents a position where a nucleotide was deleted. When the gene of the present invention is compared with the known gene, they are identical except for the following three bases:

| Base (No.) of Sequence No. 1 | Corresponding Base (No.) of Sequence No. 2 |
|------------------------------|--------------------------------------------|
| G (64)                       | A (789)                                    |
| C (224)                      | T (949)                                    |
| T (297)                      | C (1022)                                   |

As is apparent from the above comparison, these two DNA sequences are almost identical to each other. That is, the DNA prepared in the present invention is a rat synaptophysin gene.

As has been described above, according to the present invention, there is provided a monoclonal antibody specifically binding to the rat synaptophysin. By using this monoclonal antibody, only the rat synaptophysin can be easily analyzed from proteins transferred to a nitrocellulose membrane in accordance with an indirect enzyme immunoassay or indirect fluorescent antibody technique.

In addition, since the synaptophysin can serve as a good detection marker for neuroendocrine tumors, it is possible to detect neuroendocrine tumors. Therefore, the monoclonal antibody according to the present invention is expected

to be used in diagnosis in the clinical and medical fields.

# Sequence Listing

SEQ ID NO :1

SEQUENCE LENGTH :750 base pairs

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

MOLECULE TYPE:CDNA to mRNA

HYPOTHETICAL:No

ORIGINAL SOURCE

ORGANISM:Rattus norvegicus

IDENTIFICATION METHOD:S

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10      20      30      40      50      60
AGCACCTGGC GATGCCTACG GCGATGGGGG CTACGGGGCAG GGCCCCGGAG GCTATGGGCC
70      80      90     100     110     120
CCAGGACTCC TACGGGCCCTC AGGTGGTTA TCAACCCGAT TACGGGCAGC CAGCCAGCGG
130     140     150     160     170     180
TGGCGGTGGC TACGGGCCCTC AGGGCGACTA TGGGCAGCAA GGCTATGGCC AACAGGGTGC
190     200     210     220     230     240
GCCCCACCTCC TTCTCCAATC AGATGTAATC TGGTCAGTGA AGTCCATGAA GATCCCCACGG
250     260     270     280     290     300
GTGGGCAAGA GCTCAAGAGA AGGCCTGCCC CCTTTTCCC ATCCCCATAT CCTAGGTCTC
310     320     330     340     350     360
CACCCCTCAA CCCAGGAGAC CCTAACTGTC TTGCTGTTT ATATATATAT ATATTATATA
370     380     390     400     410     420
TAAATATCTA TTTATCTGTC TGAGCCCTAC ATTCACCCAC TTCTCCATGC ACTAGAGGCC
430     440     450     460     470     480
CAGTCCTGAA TGGGCTCCTC CCCAACCCCTG ACCTTGCATT CCTCAGCCCC TATCTGTTCC
490     500     510     520     530     540
CCAGCCCTGT CCCTTGAGGT AAGGGGCTCT AGAAAGGGGA GAGGAAGGGA ACCAGACCTT
550     560     570     580     590     600
GGCTGCATGG AGTGGGTGG TGTGACTTTC TCTCCTTCCT CCTCTCCCTC TGCCCCCTCT
610     620     630     640     650     660
AACTCTGGCC TTGGTCCTCC AGCATCACCT GAAC TTCAGA AGCTCTCGAA TGGAAATCTG
670     680     690     700     710     720
ACCCCAAGAG TAGAGCAGTA GACTGAGTGG AGGAGGCTTG GGTGAAACGG GCAGAGAGGA
730     740     750
GATAACCTCT GTAGAGAGAG GACTAGTCAG

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SEQ ID NO :2

SEQUENCE LENGTH :781 base pairs

SEQUENCE TYPE:nucleic acid

STRANDEDNESS:single

TOPOLOGY:linear

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721 726 736 746 756 766 776 786
CAACCAGCACCTGGC GATGCCTACG GCGATGCGGG CTACGGGCAG GCGCCCGGAG GCTATGGGCC
796 806 816 826 836 846
CCAGGACTCC TACGGGCCCTC AGGGTGGTTA TCAACCCGAT TACGGGCAGC CAGCCAGCGG
856 866 876 886 896 906
TGGCGGTGGC TACGGGCCCTC AGGGCGACTA TGGGCAGCAA GGCTATGGCC AACAGGGTGC
916 926 936 946 956 966
GCCCACCTCC TTCTCCAATC AGATGTAATC TGGTCAGTGA AGTCCATGAA GATCCACCGG
976 986 996 1006 1016 1026
GTGGGCAAGA GCTCAAGAGA AGGCCTGCCC CCCTTTTCCC ATCCCCATAT CCTAGGTCTC
1036 1046 1056 1066 1076 1086
CACCCCTCAA CCCAGGAGAC CCTAACTGTC TTTGCTGTTT ATATATATAT ATATTATATA
1096 1106 1116 1126 1136 1146
TAAATATCTA TTTATCTGTC TGAGCCCTAC ATTCACCCAC TTCTCCATGC ACTAGAGGCC
1156 1166 1176 1186 1196 1206
CAGTCCTGAA TGGGCTCCTC CCCAACCCCTG ACCTTGCAAT CCTCAGCCCC TATCTGTTC
1216 1226 1236 1246 1256 1266
CCAGCCCTGT CCCTTGAGGT AAGGGGCTCT AGAAAGGGGA GAGGAAGGGA ACCAGACCTT
1276 1286 1296 1306 1316 1326
GGCTGCATGG AGTGGGTGG TGTGACTTTC TCTCCTTCCT CCTCTCCCTC TGCCCCCTCT
1336 1346 1356 1366 1376 1386
AACTCTGGCC TTGGTCTCCTCC AGCATCACCT GAACTTCAGA AGCTCTCGAA TGGAAATCTG
1396 1406 1416 1426 1436 1446
ACCCCAAGAG TAGAGCAGTA GACTGAGTGG AGGAGGCTTG GGTGAAACGG GCAGAGAGGA
1456 1466 1476 1486 1496
GATAACCTCT GTAGAGAGAG GACTAGTCAG CCAAGAGTTG AATCCAGAC ATACT

```

## Claims

1. The monoclonal antibody produced by the hybridoma RB2-4 FERM BP-3944.
2. A pharmaceutical composition containing the monoclonal antibody of claim 1.
3. The hybridoma RB2-4 FERM BP-3944.

**Patentansprüche**

1. Monoklonaler Antikörper, hergestellt durch das Hybridom RB2-4 FERM BP-3944.
- 5 2. Pharmazeutische Zubereitung, die den monoklonalen Antikörper nach Anspruch 1 enthält.
3. Hybridom RB2-4 FERM BP-3944.

10 **Revendications**

1. Anticorps monoclonal produit par l'hybridome RB2-4 FERM BP-3944.
2. Composition pharmaceutique contenant l'anticorps monoclonal de la revendication 1.
- 15 3. Hybridome RB2-4 FERM BP-3944.

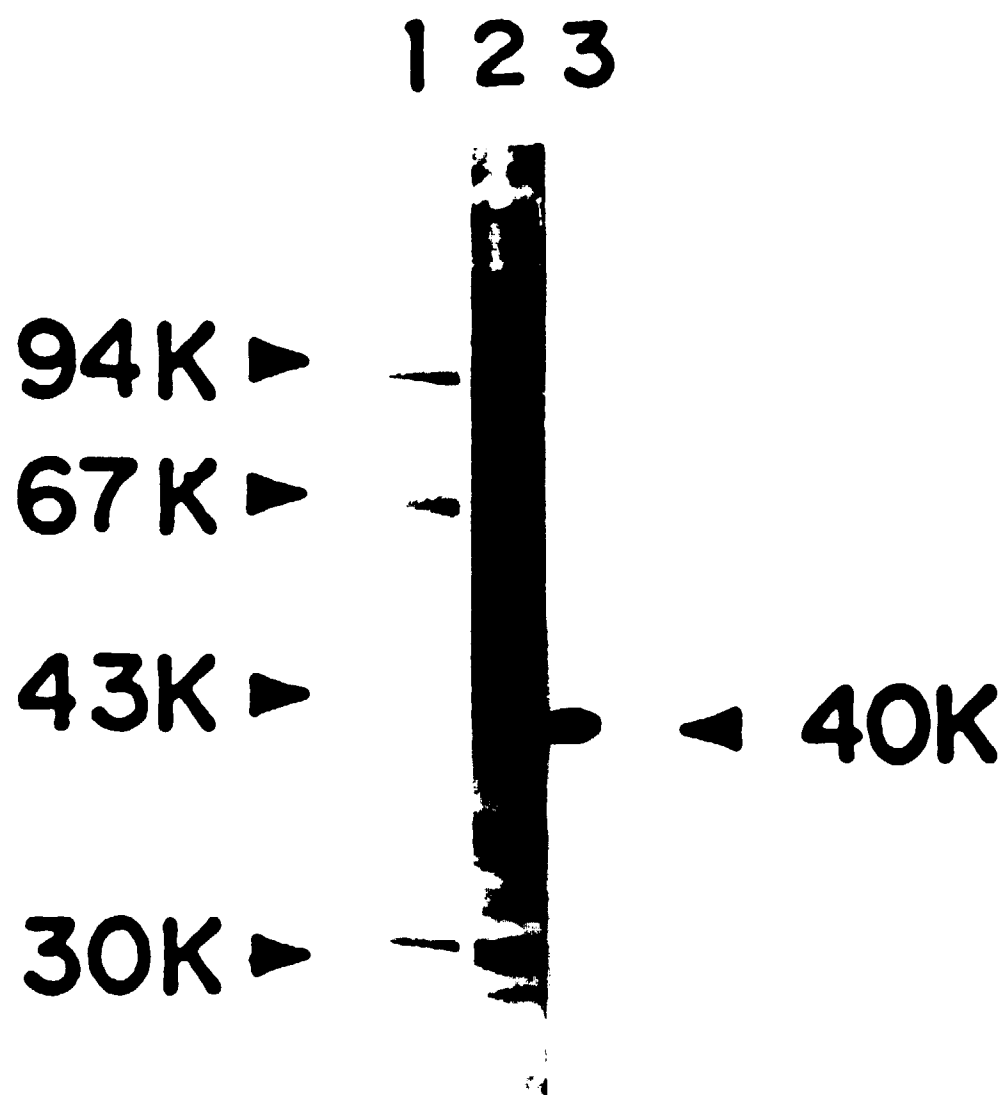


FIG. 1

1<sup>-</sup> AGCACCTGGCGATGCCTACGGCGATGCGGGCTACGGGCAGGGCCCCGGAGGCTAT  
 \*\*\*\*\*  
 721<sup>+</sup> CAACCAGCACCTGGCGATGCCTACGGCGATGCGGGCTACGGGCAGGGCCCCGGAGGCTAT  
  
 56<sup>-</sup> GGGCCCCAGGACTCCTACGGGCCTCAGGGTGGTTATCAACCCGATTACGGGCAGCCAGCC  
 \*\*\*\*\*  
 781<sup>+</sup> GGGCCCCAAGACTCCTACGGGCCTCAGGGTGGTTATCAACCCGATTACGGGCAGCCAGCC  
  
 116<sup>-</sup> AGCGGTGGCGGTGGCTACGGGCCTCAGGGCGACTATGGGCAGCAAGGCTATGGCCAACAG  
 \*\*\*\*\*  
 841<sup>+</sup> AGCGGTGGCGGTGGCTACGGGCCTCAGGGCGACTATGGGCAGCAAGGCTATGGCCAACAG  
  
 176<sup>-</sup> GGTGCGCCACCTCCTTCTCCAATCAGATGTAATCTGGTCAGTGAAGTCCATGAAGATCC  
 \*\*\*\*\*  
 901<sup>+</sup> GGTGCGCCACCTCCTTCTCCAATCAGATGTAATCTGGTCAGTGAAGTTCATGAAGATCC  
  
 236<sup>-</sup> CACGGGTGGGCAAGAGCTCAAGAGAAGGCCTGCCCCCTTTTCCCATCCCCATATCCTAG  
 \*\*\*\*\*  
 961<sup>+</sup> CACGGGTGGGCAAGAGCTCAAGAGAAGGCCTGCCCCCTTTTCCCATCCCCATATCCTAG  
  
 296<sup>-</sup> GTCTCCACCCCTCAACCCAGGAGACCCTAACTGTCTTTGCTGTTTATATATATATATATT  
 \* \*\*\*\*\*  
 1021<sup>+</sup> GCCTCCACCCCTCAACCCAGGAGACCCTAACTGTCTTTGCTGTTTATATATATATATATT  
  
 356<sup>-</sup> ATATATAAATATCTATTTATCTGTCTGAGCCCTACATTCACCCACTTCTCCATGCACTAG  
 \*\*\*\*\*  
 1081<sup>+</sup> ATATATAAATATCTATTTATCTGTCTGAGCCCTACATTCACCCACTTCTCCATGCACTAG

F I G. 2 A

416<sup>-</sup> AGGCCCAGTCCTGAATGGGCTCCTCCCCAACCTGACCTTGCATTCCTCAGCCCCTATCT  
 \*\*\*\*\*  
 1141<sup>-</sup> AGGCCCAGTCCTGAATGGGCTCCTCCCCAACCTGACCTTGCATTCCTCAGCCCCTATCT  
  
 476<sup>-</sup> GTTCCCAGCCCTGTCCCTTGAGGTAAGGGGCTCTAGAAAGGGGACAGGAAGGGAACCAG  
 \*\*\*\*\*  
 1201<sup>-</sup> GTTCCCAGCCCTGTCCCTTGAGGTAAGGGGCTCTAGAAAGGGGACAGGAAGGGAACCAG  
  
 536<sup>-</sup> ACCTTGGCTGCATGGAGTGGGTTGGTGTGACTTTCTCTCCTTCCTCCTCTCCCTCTGCCC  
 \*\*\*\*\*  
 1261<sup>-</sup> ACCTTGGCTGCATGGAGTGGGTTGGTGTGACTTTCTCTCCTTCCTCCTCTCCCTCTGCCC  
  
 596<sup>-</sup> CTCCTAACTCTGGCCTTGGTCCTCCAGCATCACCTGAACTTCAGAAGCTCTCGAATGGAA  
 \*\*\*\*\*  
 1321<sup>-</sup> CTCCTAACTCTGGCCTTGGTCCTCCAGCATCACCTGAACTTCAGAAGCTCTCGAATGGAA  
  
 656<sup>-</sup> ATCTGACCCCAAGAGTAGAGCAGTAGACTGAGTGGAGGAGGCTTGGGTGAAACGGGCAGA  
 \*\*\*\*\*  
 1381<sup>-</sup> ATCTGACCCCAAGAGTAGAGCAGTAGACTGAGTGGAGGAGGCTTGGGTGAAACGGGCAGA  
  
 716<sup>-</sup> GAGGAGATAACCTCTGTAGAGAGAGGACTAGTCAG  
 \*\*\*\*\*  
 1441<sup>-</sup> GAGGAGATAACCTCTGTAGAGAGAGGACTAGTCAGCCAAGAGTTGAATTCAGACATACT

F I G. 2B